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CD3+, CD4+ & CD8+ tumour infiltrating lymphocytes (TILs) are predictors of favourable survival outcome in infiltrating ductal carcinoma of breast

Ankita Singh Rathore[1], Sandeep Kumar[2], Rituraj Konwar[3], Annu Makker[1], M.P.S. Negi[4] & Madhu Mati Goel[1]

Department of [1]Pathology & [2]Surgery, King George's Medical University & [3]Endocrinology Division & [4]Statistics Division, Central Drug Research Institute, Lucknow, India

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***Background & objectives*: Tumour infiltrating lymphocytes (TILs) represent the host immune response against cancer cells associated with good or bad prognosis in different tumour types. This study was undertaken to evaluate the significance of CD3+, CD4+ and CD8+ TILs in breast cancer tissues in relation to clinico-pathological variables and survival outcome.**

***Methods*: Immunohistochemistry (IHC) was performed with antibodies against CD3, CD4 and CD8 antigens on formalin-fixed paraffin-embedded tissue sections of 150 breast cancer patients. Intratumoural and stromal TIL counting was performed semiquantitatively.**

***Results*: The higher CD3+, CD4+ and CD8+ intratumoural and stromal counts showed independent and direct association with good prognosis. The prognostic predictor value of intratumoural counts was higher than stromal counts. The independent associations of intratumoural and stromal counts became more prominent when adjusted with stage and grade, respectively. Among intratumoural counts, the high (++/+++) CD4+ count (OR=3.85, 95% CI=3.28-16.71, *P*<0.001) showed the highest survival followed by CD3+ (OR=2.70, 95% CI=1.76-8.30, *P*=0.001) and CD8+ (OR=2.58, 95% CI=1.55-5.86, *P*=0.001) the least when compared to respective low (+) counts. In contrast, among stromal counts, the high CD8+ count (OR=3.13, 95% CI=2.20-9.57, *P*<0.001) showed the highest survival followed by CD4+ (OR=3.02, 95% CI=2.07-8.89, *P*<0.001) and CD3+ (OR=2.45, 95% CI=1.53-6.73, *P*=0.002) the least.**

***Interpretation & conclusions*: Our results suggest that intratumoural CD4+ and stromal CD8+ counts by immunohistochemistry may serve as an independent prognosticator for favourable outcome in breast cancer.**

Key words Breast cancer - CD3 - CD4 - CD8 - ductal carcinoma - tumour infiltrating lymphocytes

Present address: [2]Director, All India Institute of Medical Sciences, Bhopal 426 026 (MP); [4]Senior Technician, Clinical & Experimental Medicine, Central Drug Research Institute, Lucknow 226 001, India

Breast cancer is a leading cause of mortality in women worldwide[1]. In India, it is the second most important cancer among females after cervical cancer[2]. Breast cancer pathogenesis is multifactorial. The traditional prognostic markers for breast cancer include tumour grade, clinical stage, lymph node with estrogen (ER), progesterone (PR) and human epidermal growth factor receptor (HER2/neu or c-erb B2) status[3].

Human cancer tissue is infiltrated by lymphocytes called tumour infiltrating lymphocytes (TILs) representing the local immune response directed against tumour growth and metastasis. These TILs have been considered as independent prognostic indicator in a number of malignant tumours[4-12]. Breast cancer tissue is invaded by a mixed population of immune cells, including T cells, B cells, natural killer (NK) cells and macrophages. Despite the existence of this immune response, many breast cancers progress and spread, questioning the function of TILs in the tumour microenvironment[13]. There is no conclusive data about the link between amount and kind of lymphocyte infiltration and the tumour growth in different kinds of breast carcinomas. These interrelationships play an important role in the pathogenesis and growth of breast cancer.

CD3 antigen is a receptor glycoprotein present on mature T lymphocytes. While high CD3+ cell density has been reported to correlate with favourable outcome in oropharyngeal cancer[14], a low CD3+ count has been shown to predict a shorter disease free survival in colon and cervical cancer[5,15]. CD4 antigen is a glycoprotein found on the surface of helper T cells, regulatory T cells, monocytes and macrophages. CD4+ T lymphocytes are an essential part of adaptive immunity. CD8 antigen is also a T cell receptor glycoprotein. Generally both CD4+ and CD8+ TILs are necessary for effective tumour elimination[9], however, there are studies which indicate that CD4+ TILs are adequate to remove cancer cells in the absence of the CD8+ TILs[16,17]. Higher CD8+ and other TILs have been shown to be associated with good prognosis in colon and ovarian carcinoma[18]. It has been also suggested that CD8+ infiltration may inhibit tumour growth[6,18]. In particular, higher number of CD8+ TILs have been linked with disease free survival and overall survival [7,18,19].

The present study was aimed to evaluate the prognostic relevance of CD3+, CD4+ and CD8+ TILs in breast cancer tissue compared to the conventional prognostic markers *viz*. tumour staging, grading, lymph node and hormone receptor status.

Material & Methods

A total of 150 histologically proven consecutive cases of breast cancer recruited between December, 2008 to October, 2011 from the Department of General Surgery, King George's Medical University, Lucknow, UP, India were included in the study after obtaining informed written consent. The study protocol was approved by the Ethics Committee of King George's Medical University, Lucknow. Demographic details, clinical history, complete general/ local examination and epidemiological risk factors including family history, clinical stage, tumour grade, lymph node status, ER, PR, and HER-2/neu were recorded on a detailed proforma especially designed for the study.

All patients had a pre-operative tissue diagnosis of breast cancer either by fine needle aspiration cytology and/or core biopsy of the breast (Fig. 1a). All patients underwent surgery with axillary lymph node dissection, and none of these patients had received pre-operative antitumour therapy. Histological grading was done by Bloom and Richardson scoring[20]. No patient had evidence of active infection or inflammatory disease. Detailed histopathological examination was done in the Department of Pathology, King George's Medical University, Lucknow, India.

Immunohistochemistry: Immunohistochemistry was performed using primary antibodies against CD3+, CD4+, CD8+, ER, PR and HER-2/neu using Novolink Min Polymer Detection system (Novacastra, Leica Biosystem Newcastle Ltd, UK). Formalin-fixed, paraffin-embedded tissues sections (3-4μm thick) were taken on 3-aminopropyltriethoxysilane (APTS) coated glass slides. Sections were deparaffinised in xylene followed by hydration in graded ethanol. Antigen retrieval was performed by heating specimens at 100°C for 20 min in 0.01M citrate buffer (*p*H 6.0) using an EZ antigen retriever system (Biogenex, USA). Endogenous peroxidase was blocked by incubating sections with 0.3 per cent hydrogen peroxide for 5 min and the non specific binding sites were blocked with a protein block for 5 min. Sections were covered with primary antibody and the slides were incubated in moist chamber overnight at 4 °C. Slides were then washed with Tris buffer saline (TBS, *p*H 7.4), followed by a 30 min incubation with post primary block at room temperature. Sections were washed twice in TBS followed by incubation with Novolink polymer for 30 min at room temperature. After three washes in TBS, sections were treated with DAB chromogen

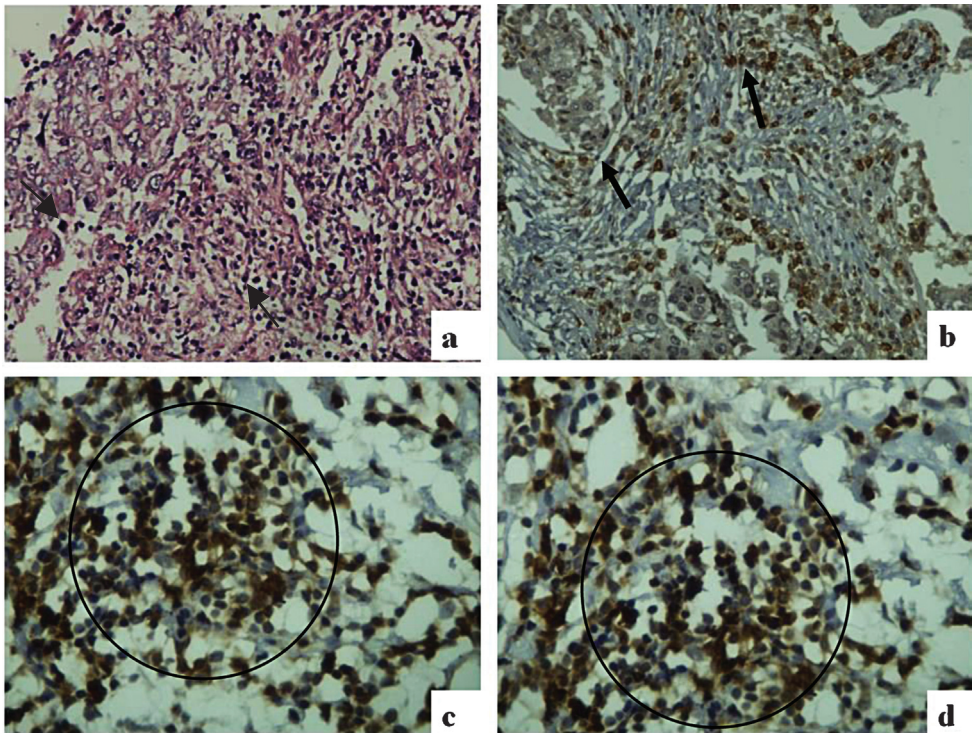

Fig. 1. (a) Histological section from infiltrating ductal carcinoma (IDC) breast showing intratumoural and stromal TILs (black arrows) (Hematoxylin & Eosin (HE) x 200); **(b)** Intratumoural and stromal CD3+ TILs in IDC (black arrows) (Immunostain x200); **(c)** Intratumoural CD4+ TILs in IDC (black circle) (Immunostain x 400); **(d)** Stromal CD8+ TILs in IDC (black circle) (Immunostain x400).

(3, 3,-diaminobenzidine tetrahydrochloride) for 5-10 min in the dark. Sections were counterstained with hematoxylin, dehydrated with ethanol and xylene, and mounted permanently with Di-n-butylPhthalate in Xylene (DPX). Negative control slides omitting the primary antibody were included in all batches. Section from tonsillar tissue served as positive control for CD3+, CD4+, CD8+.

Microscopic evaluation of CD3+, CD4+, and CD8+ TILs: Scoring of immune stained positive TILs was done independently by two pathologists. CD3+, CD4+, and CD8+ TILs were counted in five randomly selected high power fields at 40X magnification and the counts were averaged. Initially TIL count was recorded as: + (1-25 cells), ++ (26-50 cells), +++ (≥51 cells) in the tumour and the stroma separately[21]. Positive TILs upto 25 cells were considered as low TIL count and more than 25 cells (*i.e.* ++, +++) were considered as high TIL count (Fig. 1 b-d).

Microscopic evaluation of ER, PR and HER-2/neu was done as per American Society of Clinical Oncology/ College of American Pathologists Guidelines (ASCO/ CAP guidelines)[22].

ER/PR scoring was done as follows: 0 - negative, no nuclear staining; 1+ - <10 per cent nuclear staining (Borderline); 2+ - 10-75 per cent cells show nuclear staining; and 3+ >75 per cent cells show nuclear staining. At least 1 per cent of tumour cells showing positive nuclear staining of any intensity in tumour cells with antibodies to ER and PR was taken as positive.

HER-2/neu criteria for scoring was as follows: 0 - no staining or membrane staining in <10 per cent tumour cells; 1+ - faint staining in 10 per cent of cells, partial membrane staining; 2+ - a weak to moderate positive staining in >10 per cent of tumour cells; and 3+ - a strong complete membrane staining is observed in >10 per cent of tumour cells for interpretation, 0 and 1+ were considered as negative, 2 + as weak positive staining, and 3+ was considered as positive staining.

IHC result of 3+ cell surface protein expression (defined as uniform intense membrane staining of > 30 per cent of invasive tumour cells) was considered as a positive HER-2neu test. Details of clinical progress and survival of patients was obtained from the hospital records. The follow up period was 43 months.

Statistical analysis: Continuous data were summarized as mean ± SD while discrete (categorical) data were expressed in percentage. The continuous variables were compared by Student's t test and the discrete variables by Fisher's exact test or Chi-square (χ^2) test. Significance of each TIL marker with study end outcome was evaluated by binary univariate and multivariate logistic regression analysis, considering end outcome (not well=0 and well=1) as the dependent variable and TIL marker as the independent variable. Each model was adjusted with demographic confounder variables *viz*. age, menstrual status and family history. Kaplan-Meier method was used to calculate overall survival proportion and the difference of survival between the two groups was performed by log-rank test. The overall survival (OS) time was calculated for each patient to the nearest month, taken from the time of presentation to the time of death or last recorded follow up. The Kendall τ_b correlation analysis was used to assess association between the TIL markers. A two-sided (α=2) P<0.05 was considered significant. Analyses were performed using GraphPad Prism (version 5.0) (Graph Pad Software, Inc. LaJolla, CA, USA) and MINITAB (version 13.0) (Mini Tab Ltd. Conventry, UK) softwares.

Results

The basic, clinico-pathological characteristics and survival status of all patients (n=150) at admission are summarized in Table I. Age of these patients ranged from 25-86 yr with an average of 49.11 ± 12.62 yr. The menstrual cycle history was available only for 148 patients as two women had undergone hysterectomy. Majority (n=89, 59.3%) of the subjects were postmenopausal. All patients had infiltrating ductal carcinomas with 54.7 per cent showing a higher grade (III, IV), 53.3 per cent with higher stage (T3, T4) and 50.7 per cent with negative lymph node status. The survival status of only 125 (83.3%) patients was available. Of these, 83 (55.3%) were relapse free survivors, 37 (24.7%) had metastases, and 5 did not survive (Table I).

The association of each independent variable (basic, clinico-pathological characteristics and survival status) with end outcome [not well (death + recurrent disease: n=42) vs. well (relapse free survival: n=83)] are summarized in Table II. The mean age and the frequency (% age) of menstrual cycle and family history were found similar between the two groups, indicating insignificant association of these with the end outcome.

Table I. Clinico-pathological characteristics and survival status of the breast cancer patients

Characteristics	n=150 (%)
Age (yr): Mean ± SD (range)	49.11 ± 12.62 (25-86)
Menopausal status[*]	
Premenopause	59 (39.3)
Postmenopause	89 (59.3)
Grade	
I, II	68 (45.3)
III, IV	82 (54.7)
Tumour stage	
T1, T2	70 (46.7)
T3, T4	80 (53.3)
Lymph node status	
Positive	74 (49.3)
Negative	76 (50.7)
Survival status	
Relapsed	37 (24.7)
Relapse-free	83 (55.3)
Dead	5 (3.3)
Unknown	25 (16.7)

[*]Available for 148 patients only

Similarly, ER, PR and HER-2/neu, and stromal CD3+ and CD4+ counts also showed insignificant association with the end outcome. However, grading, stage and lymph node status showed significant (P<0.05 or P<0.001) association with the end outcome. Further, high CD3+, CD4+ and CD8+ intratumoural counts showed a significant (P<0.05 or P<0.01) and direct association with relapse free survival. In contrast, high count of only CD8+ stromal showed a significant (P<0.05) and direct association with relapse free survival.

To assess the independent significance of TILs with end outcome, the association of each marker was further evaluated by univariate (unadjusted or crude) and multiple (adjusted with clinico-pathological characteristics) logistic regression (LR) analysis considering the end outcome (not well=0 and well=1) as dependent variable and TILs as the independent variable. Univariate LR found all three intratumoural CD3+ (OR=2.31, 95% CI=1.02-5.26; P=0.046), CD4+ (OR=2.96, 95% CI=1.31-6.73; P=0.009) and CD8+ (OR=3.69, 95% CI=1.63-8.35; P=0.002) counts as significant (P<0.05 or P<0.01) and independent predictors of end outcome. In contrast, among stromal, CD4+ (OR=2.51, 95% CI=1.13-5.60; P=0.024) and

Table II. Association of clinico-pathological characteristics and TIL counts with end outcomes

Variables	Not well (recurrent disease + death) (n=42)	Well (relapse free survival) (n=83)	*P* value
Age (yr): Mean ± SD (range)	48.31 ± 13.17 (26-86)	49.14 ± 11.97 (25-84)	0.722
Menstrual status			
Premenopause	18 (42.9)	32 (38.6)	0.698
Postmenopause	23 (54.8)	51 (61.4)	
Family history			
No	38 (90.5)	79 (95.2)	0.440
Present	4 (9.5)	4 (4.8)	
Grading			
I-II	9 (21.4)	49 (59.0)	<0.001
III-IV	33 (78.6)	34 (41.0)	
Stage			
T1-T2	3 (7.1)	55 (66.3)	<0.001
T3-T4	39 (92.9)	28 (33.7)	
Lymph node			
Negative	14 (33.3)	47 (56.6)	0.023
Positive	28 (66.7)	36 (43.4)	
ER, PR and HER-2/neu			
000	4 (9.5)	12 (14.5)	0.799
001	5 11.9)	13 (15.7)	
010	2 (4.8)	6 (7.2)	
011	0 (0.0)	1 (1.2)	
100	1 (2.4)	2 (2.4)	
101	6 (14.3)	5 (6.0)	
110	15 (35.7)	28 (33.7)	
111	9 (21.4)	16 (19.3)	
Intratumoural CD3 count			
Low	19 (45.2)	21 (25.3)	
High	23 (54.8)	62 (74.7)	0.028
Stromal CD3 count			
Low	18 (42.9)	27 (32.5)	
High	24 (57.1)	56 (67.5)	0.324
Intratumoural CD4 count			
Low	19 (45.2)	21 (25.3)	0.028
High	23 (54.8)	62 (74.7)	
Stromal CD4 count			
Low	21 (50.0)	27 (32.5)	0.079
High	21 (50.0)	56 (67.5)	
Intratumoural CD8 count			
Low	23 (54.8)	22 (26.5)	0.003
High	19 (45.2)	61 (73.5)	
Stromal CD8 count			
Low	21 (50.0)	25 (30.1)	0.033
High	21 (50.0)	58 (69.9)	

Values in parentheses are percentages

Table III. Unadjusted and adjusted association of TIL markers with end outcomes

Markers	Adjustment with variables	Intratumoural count	Stromal count
		OR (95% CI)	OR (95% CI)
CD3	CD3 unadjusted	2.31 (1.02-5.26)*	2.04 (0.88-4.70)
	CD3 adjusted with grade	4.01 (1.49-10.79)**	3.53 (1.33-9.39)*
	CD3 adjusted with stage	11.43 (2.43-53.81)**	3.78 (1.25-11.45)*
	CD3 adjusted with node	3.08 (1.26-7.51)*	2.83 (1.14-7.07)*
	CD3 adjusted with ER, PR and HER-2/neu	2.61 (1.09-6.28)*	1.93 (0.80-4.66)
	CD3 adjusted with all#	21.37 (3.50-130.54)**	6.42 (1.68-24.61)
CD4	CD4 unadjusted	2.96 (1.31-6.73)**	2.51 (1.13-5.60)*
	CD4 adjusted with grade	6.53 (2.21-19.28)**	4.22 (1.63-10.93)**
	CD4 adjusted with stage	14.02 (2.98-65.91)**	3.36 (1.24-9.13)*
	CD4 adjusted with node	3.74 (1.55-9.02)**	3.25 (1.37-7.72)**
	CD4 adjusted with ER, PR and HER-2/neu	3.13 (1.31-7.46)*	2.26 (0.97-5.29)
	CD4 adjusted with all#	25.71 (3.99-165.59)**	5.43 (1.59-18.48)**
CD8	CD8 Unadjusted	3.69 (1.63-8.35)**	3.10 (1.32-7.29)**
	CD8 adjusted with grade	5.78 (2.19-15.28)†	5.06 (1.87-13.65)**
	CD8 adjusted with stage	16.65 (3.57-77.60)†	4.12 (1.42-11.91)**
	CD8 adjusted with node	4.77 (1.97-11.57)**	4.40 (1.72-11.26)**
	CD8 adjusted with ER, PR and HER-2/neu	3.58 (1.52-8.48)**	3.37 (1.33-8.55)*
	CD8 adjusted with all#	29.52 (4.72-184.66)†	5.66 (1.60-19.98)**

P<0.05, **<0.01, †0.001
#grade, stage, node, and ER, PR and HER-2/neu

CD8+ (OR=3.10, 95% CI=1.32-7.29; P=0.009) counts were found significant (P<0.05 or P<0.01) and independent predictors of end outcome (Table III).

The multiple LR showed that the independent associations of TILs became more significant when adjusted with risk factors (*viz*., grade, stage, node; and combination of ER, PR and HER-2/neu). Further, the insignificant stromal CD3+ count also became significant when adjusted with grade, (OR=3.53, 95% CI=1.33-9.39; P=0.011), stage (OR=3.78, 95% CI=1.25-11.45; P=0.019) and node (OR=2.83, 95% CI=1.14-7.07; P=0.026). The association of both intratumoural and stromal counts were significant when adjusted with stage and grade, respectively. On an average, with end outcome, the association of intratumoural counts was higher than the stromal counts.

The association between TIL markers is summarized in Table IV. All markers showed significant (P<0.05 or P<0.01 or P<0.001) and positive correlation with each other, with highest association seen between CD3+ stromal count and CD8+ stromal count (r=0.50, P<0.001) and least between CD4+ stromal count and CD8+ intratumoural count (r=0.20, P<0.05).

The overall survival proportion of each TIL markers according to low (+) and high (++, +++) intratumoural and stromal counts are summarized in Fig. 2. The high CD3+, CD4+ and CD8+ intratumoural counts showed significantly (P<0.01 or P<0.001) different and 2.70 (95% CI=1.76-8.30), 3.85 (95% CI=3.28-16.71) and 2.58 (95% CI=1.55-5.86) fold higher survival, respectively as compared to low intratumoural counts. Further, high CD3+, CD4+ and CD8+ stromal counts also showed significantly (P<0.01 or P<0.001) different and 2.45 (95% CI=1.53-6.73), 3.02 (95% CI=2.07-8.89) and 3.13 (95% CI=2.20-9.57) fold higher survival, respectively as compared to low stromal counts.

Discussion

The prognostic significance of tumour infiltrating lymphocytes has been a long standing topic of debate. In cases where TILs have improved patient outcome, these are recognized as the main effectors of antitumour immune responses[23]. In our study, infiltration of CD3+,

Table IV. Association between TIL markers (n=150)

TIL markers	CD3 Intratumoural count	CD3 Stromal count	CD4 Intratumoural count	CD4 Stromal count	CD8 Intratumoural count	CD8 Stromal count
CD3 Intratumoural count	1.00					
CD3 Stromal count	0.32†	1.00				
CD4 Intratumoural count	0.48†	0.28†	1.00			
CD4 Stromal count	0.40†	0.49†	0.33†	1.00		
CD8 Intratumoural count	0.32†	0.27**	0.34†	0.20*	1.00	
CD8 Stromal count	0.26**	0.50†	0.39†	0.28†	0.43†	1.00

*P**0.05, ***<0.01, †<0.001

CD4+ and CD8+ TILs was significantly associated with good prognosis. Importantly, the higher densities of CD3+, CD4+ and CD8+ TILs decreased the risk of relapse of the disease. Intratumoural CD4+ TILs were observed as better prognostic marker than CD3+ and CD8+ TILs. The observation that tumours with high TILs will have good prognosis, may not be true for all types of cancers. This may vary even within the tumour types of the same organ. Medullary carcinoma having both intratumoural and stromal CD4 or CD8 positive TILs are associated with good prognosis, but not the papillary carcinoma breast (that has only stromal)[21]. In his study on invasive micropapillary carcinoma of breast[21], TILs were found to be associated with increased lymph node metastasis and a poorer prognosis. The results suggest that effective immunity provided by TILs varies in different tumours and the relative lack of tumour-killing cytotoxic TILs in invasive micropapillary carcinoma may explain, in part, the adverse association of TILs with the biological behavior of invasive micropapillary carcinoma of breast. Matkowski *et al*[24], observed that patients with high expression of CD 4+ and CD8+ TILs had distinctly worse cancer specific overall survival. La Rocca *et al*[25] observed that the number of CD4 and CD8 expressing cells was higher in node negative than in node positive invasive ductal breast lesions. Kim *et al*[26] reported that decreased number of CD8+ TILs in breast tumours were significantly associated with lymph node metastasis, higher stage and high proliferative index.

The location of TILs, whether intratumoural or stromal is also important. In our study, higher counts of TILs were observed in both intratumoural and stromal areas of breast cancer, which were uniformly significantly associated with favourable prognosis. The densities of intratumoural TILs were found to be stronger predictor for survival than stromal TILs. A systematic review and meta analysis of 33 studies was carried out by Gooden *et al*[27] to analyse the prognostic influence of tumour-infiltrating lymphocytes in cancer. The authors however, included studies only with intratumoural TILs. In three of these studies, the CD3+ /CD8 + ratio was used, but each of them used a different interpretation of this ratio. Han *et al*[28] found an independent positive effect of either CD3+ or CD8+ compared with no CD3+ or CD8+ in ovarian cancer. In gastric cancer, it was observed that high numbers of both CD3+ and CD8+ were favourable compared with low numbers of both cell types[29]. Kobayashi *et al*[30] found that high numbers of CD8+ compared with CD3+ were not a prognostic factor in hepatocellular cancer. It is worth mentioning that while considering the CD3+ /CD8 + ratio it is important to keep in mind that most CD8+ cytotoxic lymphocytes are also CD3 positive. CD8+ /CD4+ ratio was analyzed in three studies[18,19,31], and found to be a positive prognostic predictor in only one of these[18].

Although CD4+ TILs have been shown to be sufficient to eliminate tumour cells in the absence of CD8+ T cells in some tumour models, more often both CD4+ and CD8+ are required for effective tumour rejection[16,17]. The role of CD4+ TILs in antitumour responses is often to activate the CD8+ TILs, leading to the destruction of the tumour by CD8+ cytotoxic T lymphocytes (CTL). The CD4+ T-cells help the CD8+ CTLs in tumour immunity through three phases, *viz.* by early induction, effector maintenance, and memory of CD 8+ CTL responses.

In the present study, CD3+, CD4+ and CD8+ TIL counts were not significantly associated with age, menopausal status and family history. A significant association of higher tumour grade and clinical stage with the severity of disease was found. Lymph node positivity was also correlated with the relapse of breast

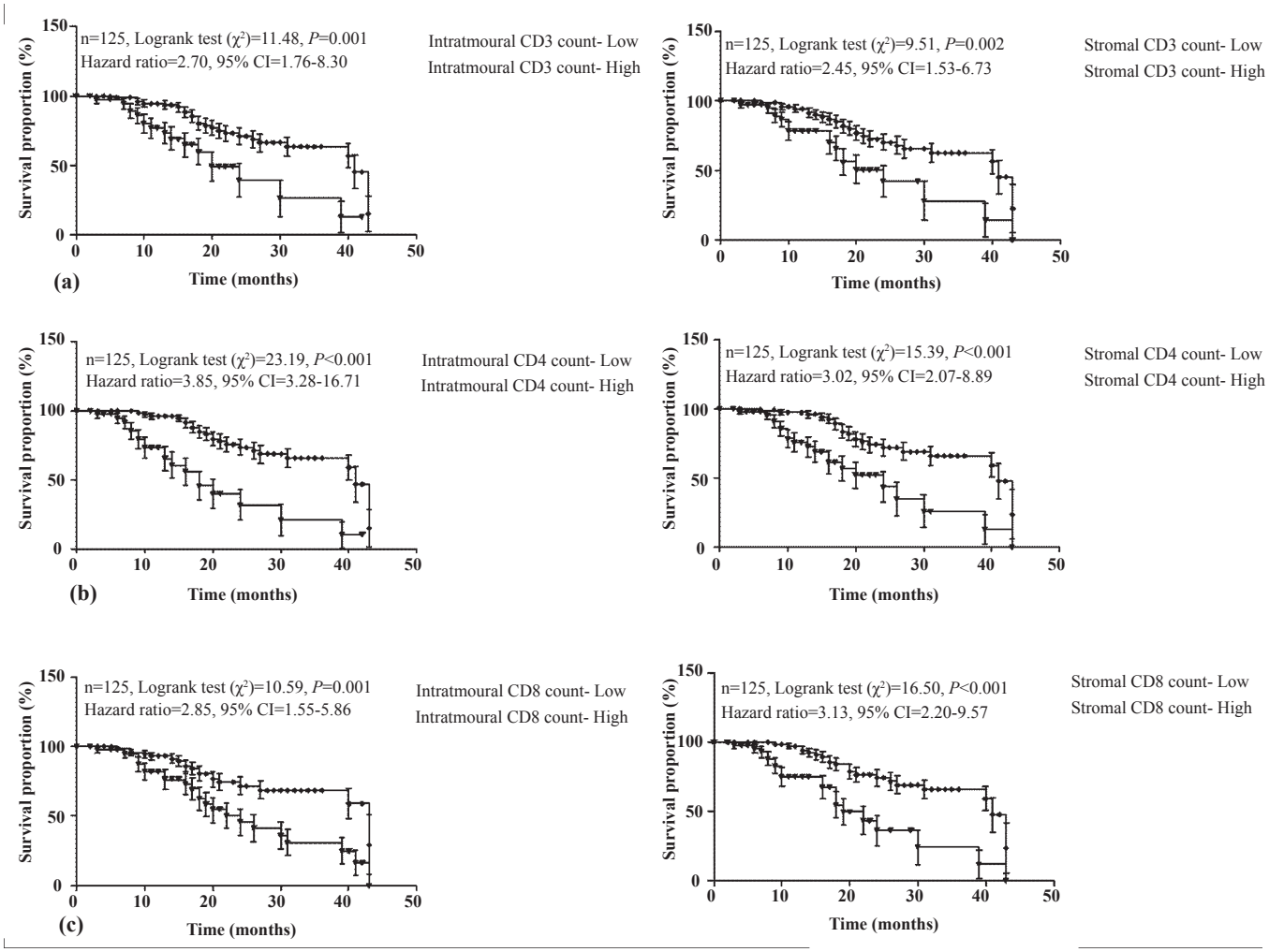

Fig. 2. Overall survival proportions according to CD3+ **(a)**, CD4+ **(b)** and CD8+ **(c)** intratumoural and stromal TIL counts, respectively.

cancer. As all the assessed cases in the present study were of infiltrating ductal carcinomas (NOS), the prognostication cannot be generalized for all subtypes of breast cancer. An increase in TILs may not always be associated with a better prognosis. There is evidence in the literature to indicate that tumour specific CD4+ TILs may change their phenotype from effectors to suppressors during cancer progression. Conversion from effector cells may coincide with a substantial reduction in the antigen expression level, resulting in tumour persistence that ultimately leads to tumour tolerance. This negative regulatory role of CD4+ TILs needs to be distinguished from the conventional role of activated CD4+ TILs[23].

To conclude, CD3+, CD4+ and CD8+ TILs were found to be good prognosticators of overall survival of infiltrating ductal carcinoma of breast. Although quantifying TILs by histopathology and immunohistochemistry is a frequently used approach, the methodological factors may confound the exact magnitude of TILs on prognosis. Moreover, just quantifying TILs may not take into account the dynamics and functionality of the tumour microenvironment. The study needs to be done in various histological subtypes of breast cancer, in a larger sample size, including the cytokines or cytotoxicity assay along with molecular signatures for a better understanding of role of TILs[27].

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Reprint requests: Dr Madhu Mati Goel, Department of Pathology, King George's Medical University, Lucknow 226 003, India
e-mail: madhukgmc@gmail.com